SURGERY FOR ACQUIRED HEART DISEASE

MECHANISMS UNDERLYING DEGENERATION OF CRYOPRESERVED VASCULAR HOMOGRAFTS

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Objective: **To analyze the mechanism(s) underlying homograft degeneration, we designed an experimental model in which the behavior of cryopreserved autografts and homografts, as well as fresh autografts, implanted in the same animal was compared.** ***Methods:*** **A cryopreserved homograft was implanted in the aorta of 14 sheep. The excised aortic autologous segment was then subjected to cryopreservation, and 1 to 8 weeks later it was implanted 1 to 2 cm below the cryopreserved homograft. The intermediate segment of the native aorta, the fresh autograft, was dissected at this point. Animals were put to death at different times and the implanted segments were harvested together with a portion of native aorta. Histologic and immunohistochemical analyses, as well as cell viability assessments, were then performed on the explanted segments. Similar studies were also conducted on fragments of cryopreserved autografts and homografts before implantation.** ***Results:*** **With the exception of a partial loss of the endothelium, cryopreserved specimens retained cell viability and morphologic integrity before implantation. Explanted cryopreserved homografts showed profound changes affecting all strata, as well as a decline in cell viability. Lymphocyte infiltrates were found up to 12 months after implantation. Endothelium was always absent in cryopreserved homografts. However, a reendothelialization of the cryopreserved autografts was observed. After an initial period of neuronal degeneration, reenervation of the cryopreserved autograft segment occurred 6 to 12 months after the operation. Findings regarding the fresh autografts were similar to those of the cryopreserved autografts.** ***Conclusion:*** **Our results suggest that the immunologic reaction rather than the cryopreservation process is responsible for the degenerative process occurring in cryopreserved homografts. (J Thorac Cardiovasc Surg 1997;113:1014-21)**

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Cryopreserved homograft heart valves and conduits are frequently used in the surgical correction of several diseases, in which they remain the substitutes of choice. However, the mechanisms underlying the degenerative process that ultimately leads to their failure remain unknown.

Recent studies comparing heart valve homografts and valves of transplanted hearts showed that whereas the latter contained fibroblasts of both donor and recipient origin, the former were acellular.[1-3] These differences could be due to either the occurrence of an immune response in homograft valve recipients, which was prevented or abrogated by the immunosuppressive therapy administered to heart transplant recipients but not to homograft valve recipients, or to the cryopreservation process

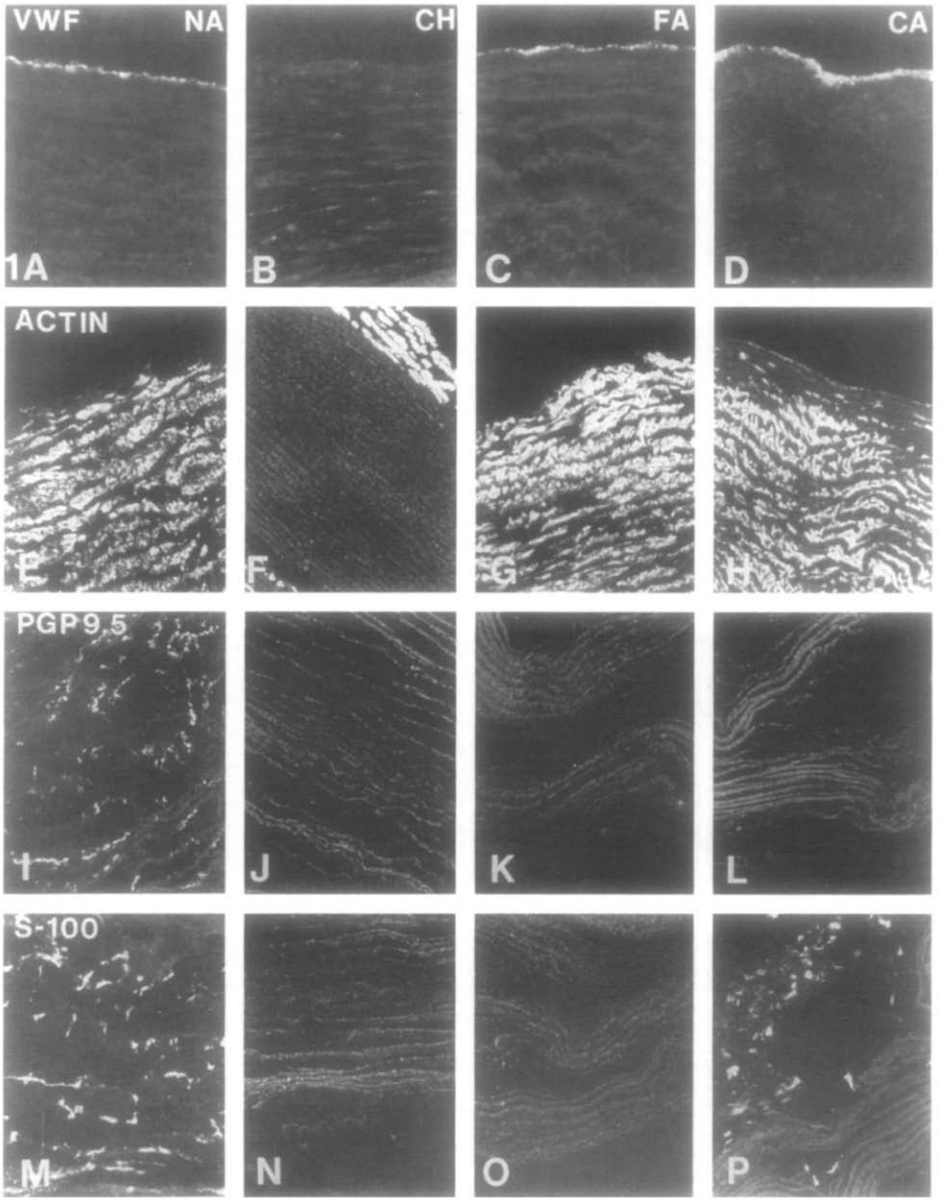

Fig. 1. Cryostat sections of sheep native aorta (*NA;* parts **A, E, I,** and **M**), fresh autografts (*FA;* parts **C, G, K,** and **O**), and aortic cryopreserved homografts (*CH;* parts **B, F, J,** and **N**) and autografts (*CA;* parts **D, H, L,** and **P**) 3 months after implantation in the same animal. Preparations were immunostained for the von Willebrand factor (*VWF;* parts **A, B, C,** and **D**), ∝-smooth muscle actin (*ACTIN;* parts **E, F, G,** and **H**), protein gene product 9.5 (*PGP9.5;* parts **I, J, K,** and **L**), and S-100 (parts **M, N, O,** and **P**). (**A, B, C,** and **D**, original magnification ×272; **E** through **P,** original magnification ×136.)

to which the homograft valves were subjected. To distinguish between these two alternatives, we designed an experimental model in which the behavior of cryopreserved autografts and homografts, implanted in the same animal, was compared. Fresh autografts were used to analyze the role of denervation and devascularization. The biologic alterations induced by the different procedures were assessed by evaluation of cell viability and by immunohistochemical analysis of the endothelium, perivascular innervation, and medial smooth muscle cells. Genetic profiles of

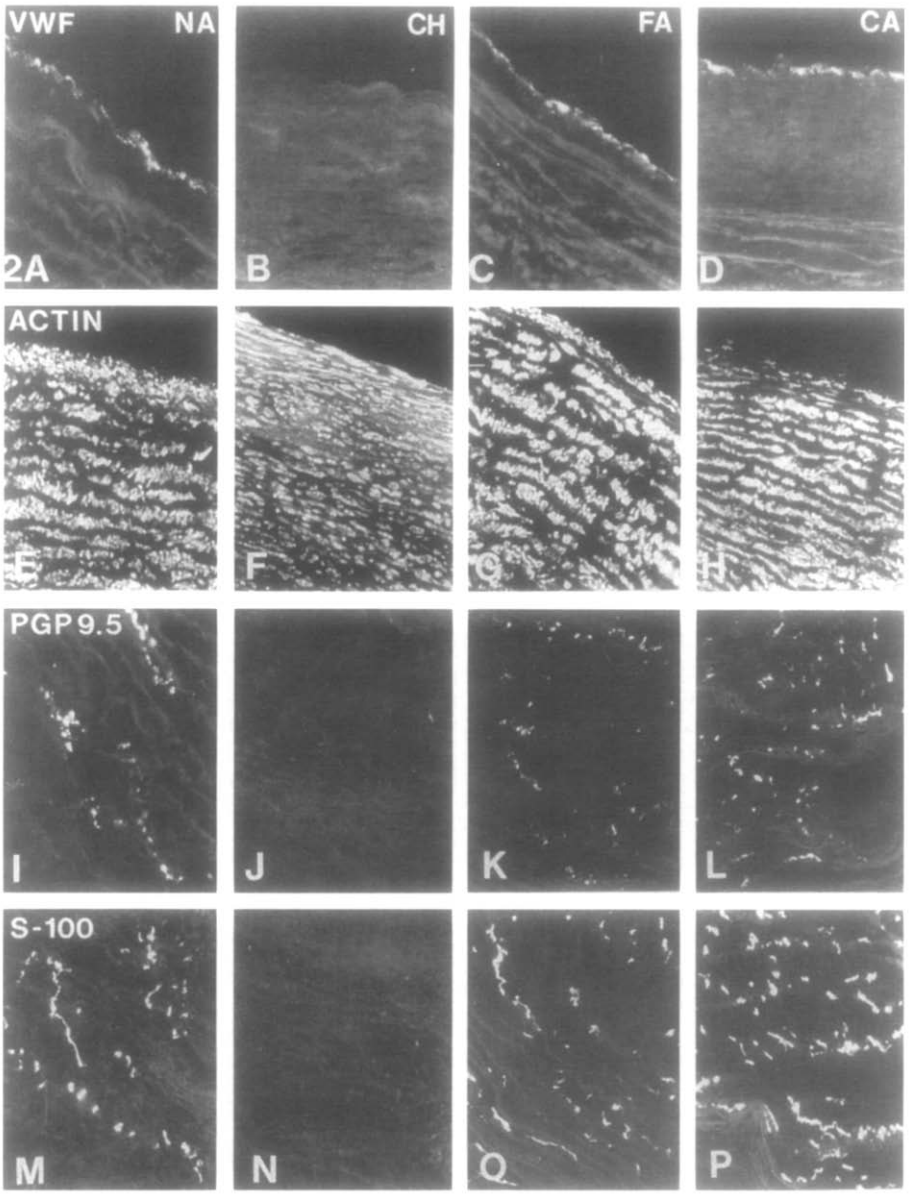

Fig. 2. Cryostat sections of sheep native aorta (*NA;* parts **A, E, I,** and **M**), fresh autografts (*FA;* parts **C, G, K,** and **O**), and aortic cryopreserved homografts (*CH;* parts **B, F, J,** and **N**) and autografts (*CA;* parts **D, H, L,** and **P**) 2 years after implantation in the same animal. Preparations were immunostained for the von Willebrand factor (*VWF;* parts **A, B, C,** and **D**), ∝-smooth muscle actin (*ACTIN;* parts **E, F, G,** and **H**), protein gene product 9.5 (*PGP9.5;* parts **I, J, K,** and **L**), and S-100 (parts **M, N, O,** and **P**). (**A, B, C,** and **D,** original magnification ×272; **E** through **P,** original magnification ×136.)

donor and recipient cells were used to determine the origin of the cells in explanted cryopreserved homografts.

Material and methods

Animals. Fifteen Merino Branco sheep, eight ewes and seven rams, aged 2 to 18 months, were used.

Surgery. Standard anesthesia technique with thiopental sodium (Pentothal) and halothane was used. Humane care was provided to all animals during the experiment, in compliance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985). Cryopreserved homografts of the descending thoracic aorta were prepared as previously described.[1, 2] In the first operation, a 1 to 2 cm segment of thoracic aorta was replaced by a cryopreserved homograft. The excised aortic segment was then subjected to the same cryopreservation process used for treatment of the homografts. One to 8 weeks later, the cryopreserved autograft was im-

planted 1 to 2 cm below the cryopreserved homograft. The intermediate segment of the native aorta was, at this point, completely dissected from the surrounding tissues and used as a fresh autograft. Animals were put to death at different times (2 weeks, 1, 3, 6, 12, and 24 months) after the second operation and the implanted segments were harvested together with a portion of native aorta to be used as control tissue. With regard to the latter segment, care was taken to analyze areas that were far from the surgical sites.

Immunohistochemistry. Immediately after excision, the various segments were fixed by immersion according to the method of Steffanini, De Martino, and Zamboni[4] for 18 to 24 hours at 4° C. After being rinsed in several changes of phosphate-buffered saline solution (0.01 mol/L, pH 7.2) containing 15% sucrose, tissues were processed as cryostat sections for indirect immunofluorescence staining together with antisera raised against ∝-smooth muscle actin and neuronal markers (protein gene product 9.5), endothelial markers (von Willebrand factor), and Schwann cell markers (S-100). Primary antisera were obtained from Sigma Chemical Company, St. Louis, Missouri, except for the antiserum to protein gene product 9.5, which was obtained from Ultraclone, Isle of White, United Kingdom.

Cell viability. Initially, cell viability was evaluated by culturing tissue fragments in 10 ml flasks for 3 to 4 weeks in Dulbecco's modified medium at 37° C in an atmosphere containing 10% carbon dioxide and graded as 0 to 5, according to the rate of fibroblast growth. Explanted fragments were also analyzed by a new method, devised to ascertain a less subjective evaluation of cell viability. Thus 8 to 12 fragments of 1 mm diameter, excised from each portion of the explanted conduit, were cultured in RPMI medium (Roswell Park Memorial Institute, Buffalo, N.Y.) containing 10% fetal calf serum, in 96-well microtiter plates, at 37° C in an atmosphere containing 5% carbon dioxide. Fibroblast growth was evaluated microscopically on days 4, 8, and 15 after initiation of cultures. Wells with five or more fibroblasts were considered positive. The results were then expressed in percent of positive wells.

Analysis of DNA hypervariable regions. Cell origin was determined as previously described[1, 2] by analyzing the hypervariable (CA)n repeat regions from DNA of native aorta and cryopreserved homografts. In brief, DNA obtained from tissues by the standard proteinase K method, followed by phenol-chloroform extraction, was amplified by polymerase chain reaction with the use of oligonucleotide primers for TGLA53, TGLA126, TGLA263, MGTG7, and MGTG4B loci (kindly provided by Applied Biosystems Division of Perkin-Elmer, Foster City, Calif.). The polymerase chain reaction amplification was performed in a model PE 9600 thermocycler and the products were electrophoresed in an automated DNA sequencer (model 373A, ABI Analytical). The size of the (CA)n amplimers was determined with the use of the GENESCAN 672 software (ABI Analytical).

Results

With the exception of a partial loss of endothelial cells, cryopreserved specimens retained cell viability and morphologic integrity before implantation.

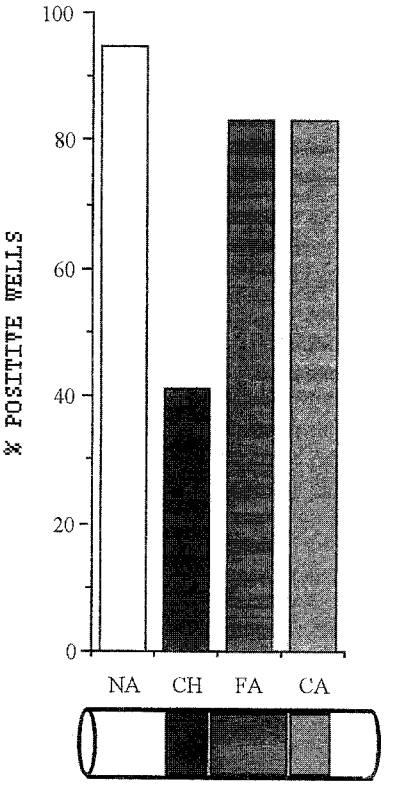

Fig. 3. Cell viability expressed in percent positive wells in native aorta *(NA)*, cryopreserved homograft *(CH)*, fresh autograft *(FA)*, and cryopreserved autograft *(CA)* explanted 2 years after implantation. Below the x-axis a schematic representation of the fragments, as they were implanted, is shown.

The results obtained with the various markers 3 months and 2 years after the last operation are shown in Figs. 1 and 2, respectively. These two time points illustrate the degenerative and regenerative processes that occurred during the course of the experiment (2 years). As can be seen, explanted cryopreserved homografts showed profound histologic changes that affected all strata, as well as decline in cell viability (Fig. 3). After an initial period of nonspecific inflammatory reaction, which in most cases subsided after 1 to 3 months, progressive neuronal and smooth muscle degeneration, as well as fragmentation of elastic fibers, was observed, leading in later stages (6 to 12 months) to the disappearance of the perivascular innervation, fibrosis hyalinization, and calcification. Most likely as a result of this process, one cryopreserved homograft ruptured after 17 months. Polymorphonuclear cells disappeared after 1 to 3 months; lymphocyte infiltrates, however, persisted up to 2 years after implan-

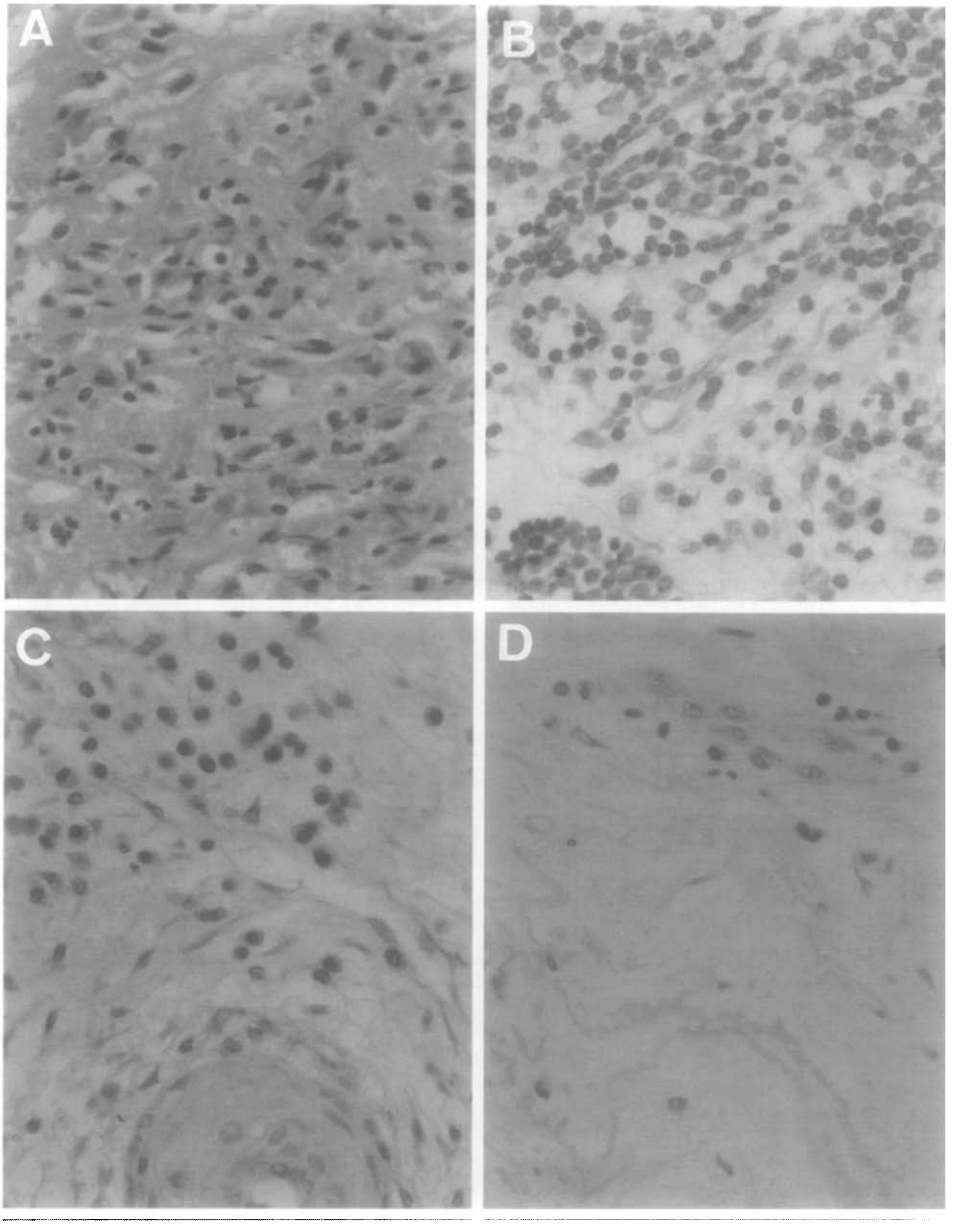

Fig. 4. In allografts, lymphocyte infiltrates persisted up to 2 years after implantation. Allografts explanted at 8 days (**A**), 15 days (**B**), 1 month (**C**), and 2 years (**D**) show an initial period of nonspecific inflammatory reaction with polymorphonuclear cell infiltrates (**A** and **B**). After 1 to 3 months only lymphocytes were observed (**C** and **D**). (Hematoxylin and eosin stain; original magnification ×400.)

tation (Fig. 4). Although endothelial cells were always absent in cryopreserved homografts, reendothelialization occurred in the cryopreserved autografts. After an initial inflammatory reaction, as in all other segments, nerve degeneration was observed in cryopreserved autografts. At this point (15 to 30 days), signs of smooth muscle alteration were also present, as judged by the weakness of the immunoreactive staining for actin. At 3 to 6 months and thereafter, smooth muscle cells and myofibrils had, however, recovered their normal structure, and progressive reenervation was observed, with reestablishment of the normal nervous tissue pattern being achieved 6 to 12 months after the operation. Histologically, a single alteration,

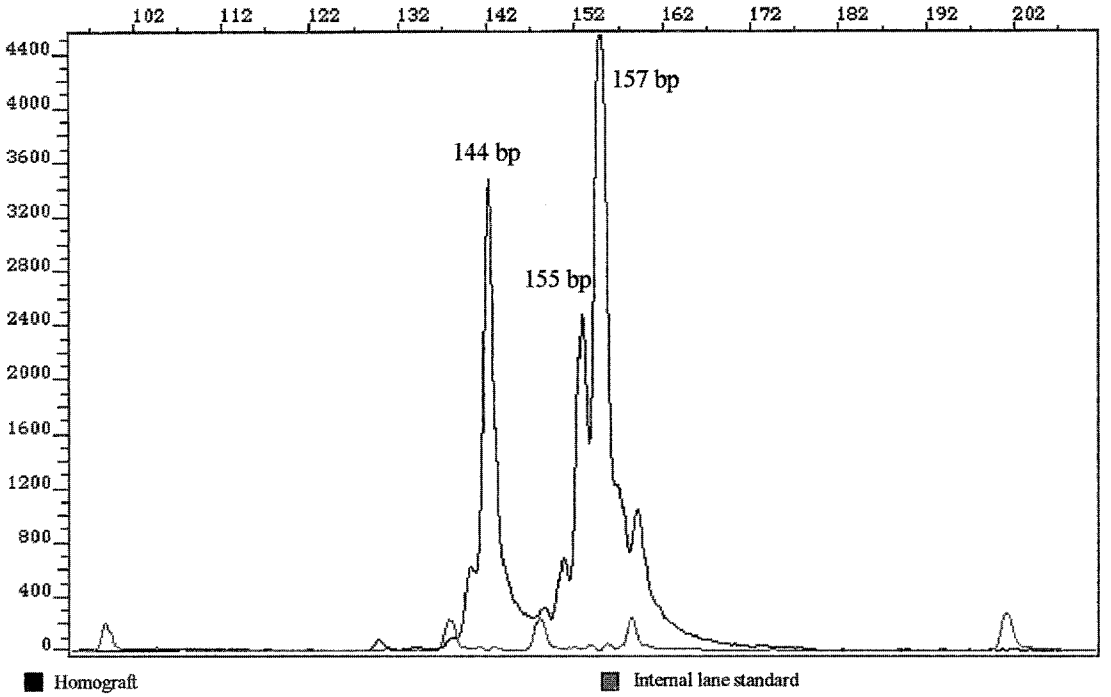

Fig. 5. Electrophoretogram of the polymerase chain reaction products with primers for TGLA53 of a homograft (case 25) explanted after 1 year. The *abscissa* indicates the size in base pairs *(bp)* and the *ordinate* indicates the fluorescence intensity.

Table I. *Origin of cells of cryopreserved homografts*

			Markers and sizes																
	Implantation time		*TGLA263*			*TGLA53*						*TGLA126*			*MGTG7*			*MGTG4B*
Sheep	*(yr)*	*Tissue*	*121*	*127*	*129*	*144*	*146*	*149*	*155*	*157*	*161*	*127*	*133*	*135*	*274*	*277*	*280*	*132*
1	2	Receptor		5096	8159				86757				31786		72771			3585
		Donor	11900*				25250	38729					7111	9738	461228			11804
		Homograft		3040	3641		24397				19262		5225					15478
2	1	Receptor	1348		3734				28414	44789		3628				7683		501
		Donor	2523		613	17969							5271			6248		450
		Homograft	1591		1308	28017			16270	35275		535	429			1492		2723

*Peak area.

consisting of an intimal thickening, was patent in these explants (see Fig. 2, *D*). Cell viability was similar to that of native aorta.

With the exception of the thickening of the intima, the loss of endothelium, and the initial alteration of the smooth muscle cells, which did not occur in fresh autografts, the histologic and immunohistochemical findings in this fragment were similar to those of the cryopreserved autografts with regard to the kinetics of neuronal degeneration and regeneration.

Origin of the cells in the cryopreserved homografts was determined in fragments explanted 1 and 2 years after the operation. The results from these experiments showed that cells of both donor and recipient origin were present in the tissue in the first animal; and in the second only the latter were found (Table I and Fig. 5).

Comment

Even when performed under optimal conditions, harvesting, handling, cryopreservation, and antibi-

otic treatment of homografts affects the degree of cell survival, the metabolic rates of the viable cells, and, consequently, matrix degradation and synthesis.[5-14] Therefore, and despite the abundant evidence of immunogenicity of the valve homograft,[1-3, 15-27] the concept that the durability of a homograft is related to the degree of cell viability at the time of implantation prevailed.[28-30]

By creating conditions in which autologous tissue was treated in the same way as homologous tissue, we could ascertain that the preparation procedures, including ischemic time, antibiotic treatment, and cryopreservation, were not responsible for the long-term deterioration found in homografts.

Of particular interest were the findings that loss of the endothelium and degeneration of the neuronal tissue smooth muscle and vasa vasorum occurred in the cryopreserved autografts as it did in the homografts. Thus the evidence that these processes were followed by regeneration in the former but not the latter further strengthens the concept that the mechanism underlying the failure of the homografts is most likely immunologic rather than physical. Whether the immune response induced by the allogeneic tissue led to the destruction of progenitor cells or prevented the release of angiogenic and neurogenic factors required for the regeneration process remains to be determined.

More information on the origin of the cells in the homograft is needed before conclusions can be drawn. However, it is possible that at 2 years the donor cells have been destroyed and replaced by recipient cells. These findings are not consistent with those obtained in human heart valves.[1-3] This discrepancy might be due to the structural differences between the two tissues, in particular the presence of muscular tissue in the aorta but not in the valve leaflets.

Some of the findings in the present study may be clinically relevant. Thus it became clear that denervation and devascularization did not affect the vitality of the tissue, inasmuch as regeneration of both systems could occur, provided factors such as the immune response did not hinder the process. Such is the case of pulmonary autografts in the aortic root position (Ross operation). Also worth notice was the single alteration found in the cryopreserved autologous grafts, the thickening of the intima. Similar findings have been reported for cryopreserved rat aortic isografts.[26] Because intimal thickening did not occur in the fresh autologous fragments, it is tempting to hypothesize that this is the sole transformation induced by cryopreservation.

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REFERENCES

1. Melo JQ, Monteiro C, Neves J, Santos R, Martins A, Ramos S, et al. The allograft valve in heart transplantation and valve replacement: genetic assessment of the origin of the cells by means of deoxyribonucleic acid profiles. J Thorac Cardiovasc Surg 1995;109:218-23.
2. Neves J, Monteiro C, Santos R, Martins A, Ramos S, Ramos T, et al. Histological and genetic assessment of explanted allograft valves. Ann Thorac Surg 1995;60:5141-5.
3. Schoen FJ, Mitchell RN, Jonas RA. Pathological considerations in cryopreserved allograft heart valves. J Heart Valve Dis 1995;4(Suppl 1):S72-6.
4. Steffanini M, De Martino C, Zamboni L. Fixation of ejaculated spermatozoa for electron microscopy. Nature 1967;216: 173-4.
5. Reichenbach DD, Mohri H, Sands M, Merendino KA. Viability of connective tissue cells following storage of aortic valve leaflets. J Thorac Cardiovasc Surg 1971;62:690-5.
6. Gavin JB, Monro JL, Wall FM, Chalcroft SCW. Fine structural changes in fibroblasts of canine heart valves prepared for grafting. Thorax 1973;28:748-55.
7. Hu J-F, Gilmer L, Hopkins R, Wolfinbarger L Jr. Effects of antibiotics on cellular viability in porcine heart tissue Cardiovasc Res 1989;23:960-4.
8. Adam M, Hu JF, Lang P, Wolfinbarger L Jr. Cryobiology 1990;27:605-14.
9. Messier RH, Domkowski PW, Aly H, et al. J Surg Res 1992;52:483-8.
10. Brokbank KGM, Dawson PE. Cytotoxicity of amphotericin B for fibroblasts in human heart valve leaflets. Cryobiology 1993;30:19-24.
11. Domkowski PW, Messier RH, Crescenzo DG, et al. Preimplantation alteration of adenine nucleotides in cryopreserved heart valves. Ann Thorac Surg 1993;55:413-9.
12. Mitchell RN, Jonas RA, Schoen FJ. Structure-function correlations in cryopreserved allograft cardiac valves. Ann Thorac Surg 1995;60:S108-13.
13. Yankah AC, Wottge H-U, Muller-Hermelink HK, Feller AC, Lange P, Wessel U, et al. Transplantation of aortic and pulmonary allografts, enhanced viability of endothelial cells by cryopreservation, importance of histocompatibility. J Card Surg 1987;2(Suppl):209-20.
14. Lupinetti FM, Tsai TT, Kneebone JM. Endothelial cell replication in an in vivo model of aortic allografts. Ann Thorac Surg 1993;56:237-41.
15. Yankah AC, Feeler AC, Thiede A, Westphal E. Identification of surface antigens of endothelial cells of fresh preserved heart allografts. Thorac Cardiovasc Surg 1986;34:97.
16. Yacoub M, Sutters A, Khagani A., Rose M. Localization of major histocompatibility complex (HLA and DR) antigens in aortic homografts. In: Bodnar E, Yacoub M, editors. Biologic

and bioprosthetic valves. Proceedings of the Third International Symposium. New York: Yorke Medical Books, 1986.
17. Tector AJ, Boyd WC, Korns ME. Aortic valve allograft rejection. J Thorac Cardiovasc Surg 1971;62:592-601.
18. Kwong K-H, Paton BC, Hill RB. Experimental use of immunosuppression in aortic valve homografts and heterografts. J Thorac Cardiovasc Surg 1967;54:199-212.
19. Yankah AC, Wottge HU, Muller-Ruchholtz W. Prognostic importance of viability and a study of a second set allograft valve: an experimental study. J Card Surg 1988;3:263-70.
20. Heslop BF, Wilson SE, Hardy BE. Antigenicity of aortic valve allografts. Ann Surg 1973;177:301-67.
21. El Khatib H, Lupinetti F. Antigenicity of fresh and cryopreserved rat valve allografts. Transplantation 1990; 49:765-67.
22. Gonzalez-Lavin L, Bianchi J, Graf D, Amini S, Gordon CI. Homograft valve calcification: evidence of an immunologic influence. In: Yankah AC, Hetzer R, Yacoub MH, editors. Cardiac valve allografts 1962-1987: current concepts on the use of aortic and pulmonary allografts for heart valve substitutes. Darmstadz: Steinkopff Verlag, 1988:69-74.
23. Buch WS, Kosek JC, Angell WW. The role of rejection and mechanical trauma in valve graft viability. J Thorac Cardiovasc Surg 1971;62:696-706.
24. Zhao X, Green M, Frazer IH, Hogan P, O'Brien MF. Donor-specific immune response after aortic valve allograft in rat. Ann Thorac Surg 1994;57:1158-63.
25. Yankah AC, Wottge HU, Müller-Ruchholtz W. Antigenicity and fate of cellular components of heart valve allografts. In: Yankah AC, Hetzer R, Yacoub MH, editors. Cardiac valve allografts 1962-1987: current concepts on the use of aortic and pulmonary allografts for heart valve substitutes. Darmstadz: Steinkopff Verlag, 1988:77-8.30.
26. Motomura N, Imakita M, Yutani C, Takamoto S, Kitoh Y, Tsuji T, et al. Histologic modification by cryopreservation in rat aortic allografts. Ann Thorac Surg 1995;60:S168-71.
27. Plissonnier D, Nochy D, Poncet P, Mandet C, Hinglais N, Bariety J, et al. Sequential immunological targeting of chronic experimental arterial allograft. Transplantation 1995; 60:414-24.
28. O'Brien MF, Stafford G, Gardner M, Pohlner P, McGiffin D, Johnston N, et l. The viable cryopreserved allograft aortic valve. J Card Surg 1987;2(Suppl):153-67.
29. Yacoub M, Rasmi NR, Sundt TM, Lund O, Boyland E, Radley-Smith R, et al. Fourteen-year experience with homovital homografts for aortic valve replacement. J Thorac Cardiovasc Surg 1995;110:186-94.
30. O'Brien MF, Stafford EG, Gardner MA, Pohlner PG, Tesar PJ, Cochrane AD, et al. Allograft aortic valve replacement: long-term follow-up. Ann Thorac Surg 1995;60:S65-70.

Discussion

Dr. Patrick G. Hogan *(Brisbane, Australia).* I am asking my questions from the perspective of an immunologist. First, were you transplanting across a major histocompatibility complex barrier? What is known about the immunogenetics of the major histocompatibility complex in sheep?

Dr. Neves. Yes. We were transplanting across the major histocompatibility complex.

Dr. Hogan. The sheep are not likely to be inbred. They would be outbred. Might they be polymorphic for major histocompatibility complex loci?

Dr. Neves. Yes, but the same condition occurs in human beings.

Dr. Hogan. I am interested in the S-100 staining. A recent paper from St. Vincent's Hospital in Australia, discussing the use of S-100, purports to show that there are dendritic cells in the human aorta. If immunologic responses are important, do you think any of your staining could have been produced by dendritic cells? I do note that there was no staining in the cryopreserved tissue.

Dr. Neves. I am a surgeon, not an immunologist, and the information that I have from my colleagues is that S-100 stains the Schwann cells. I cannot answer that.

Dr. Hogan. In your abstract, lymphocytic infiltrates are documented as one point in favor of an immune response. Could you expand on that? Were the lymphocytic infiltrates different in intensity or nature in the homografts as opposed to the autografts?

Dr. Neves. All of the segments in the first weeks have an inflammatory reaction. After 1 or 2 months, the homograft persists with lymphocytes. We tried to mark them for T cells, B cells, and activations, but we had problems with the specific markers for sheep. It was difficult to get conclusions. However, we have performed these studies in human beings and we know that there is an immunologic response with activated T cells.